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# Identification and Purification of Human Stat Proteins Activated in Response to Interleukin-2

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## Summary

**A key cytokine induced during the immune response is IL-2. Following T cell activation, the genes encoding IL-2 and the various chains of its receptor are transcriptionally induced. In turn, secreted IL-2 serves to stimulate the proliferation and differentiation of T lymphocytes. Several recent studies have implicated Jak kinases in the signaling pathway induced by IL-2. Following this lead, we set out to identify transcription factors induced in response to IL-2. Human peripheral blood lymphocytes were observed to contain several IL-2-inducible DNA binding activities. Similar activities were also observed in a transformed human lymphocyte line, termed YT. We have purified these activities and found that the principal IL-2-inducible component bears significant relatedness to a prolactin-induced transcription factor first identified in sheep mammary gland tissue. We hypothesize that activation of this protein, designated hStat5, helps govern the biological effects of IL-2 during the immune response.**

## Introduction

Acting through a high affinity multisubunit receptor, interleukin-2 (IL-2) stimulates the proliferation and differentiated function of hematopoietic cells (Taniguchi et al., 1986; Smith, 1988; Taniguchi and Minami, 1993; Waldmann, 1993). Considerable progress has been made during the past decade on the biological effects of IL-2 as well as the molecular composition of the cytokine and its cognate receptor. Cells bearing the functional receptor complex respond to IL-2 via at least two distinctive pathways. They commonly enter a proliferative state facilitating significant autocrine expansion. IL-2 can also cause receptor-bearing lymphocytes to mature along various differentiative pathways, including those leading to cytolytic and immune helper cell function. The intracellular signaling and gene regulatory circuitry controlled by IL-2 is, however, only beginning to be resolved. Perhaps the most useful information pertaining to the latter challenge has come from studies of the IL-2 receptor.

Functional IL-2 receptor is a heterotrimeric complex composed of polypeptide chains designated IL-2Rα, IL-2Rβ, and IL-2Rγ (Cosman et al., 1984; Leonard et al., 1984; Nikaido et al., 1984; Hatakeyama et al., 1989; Takeshita et al., 1992; Voss et al., 1993). The IL-2Rα chain appears to be uniquely dedicated to IL-2 response, whereas the IL-2Rβ and IL-2Rγ chains are commonly used by other cytokine receptors (Kondo et al., 1993; Noguchi et al., 1993; Giri et al., 1994). On its own, IL-2Rα is capable of low affinity binding to IL-2. High affinity binding and biological response to IL-2 requires, however, association of IL-2Rα with the IL-2Rβ and IL-2Rγ chains of the receptor (Minamoto et al., 1990; Takeshita et al., 1990). Moreover, functional signaling via the IL-2 receptor is dependent upon the integrity of the intracellular domains of IL-2Rβ and IL-2Rγ (Kawahara et al., 1994; Nakamura et al., 1994; Nelson et al., 1994).

Two lines of evidence have indicated that IL-2 may alter the program of gene expression in receptor-bearing cells via a pathway involving Janus kinases (Jaks) and signal transducers and activators of transcription (Stat). Upon stimulation by IL-2, receptor-bearing cells have been shown to activate the Jak1 and Jak3 tyrosine kinases (Boussiotis et al., 1994; Miyazaki et al., 1994; Russell et al., 1994). IL-2 has also been observed to activate an otherwise latent DNA binding activity bearing properties related to Stat proteins (Gilmour and Reich, 1994; Beadling et al., 1994). In this study, we have purified and identified the transcription factors activated in human lymphocytes in response to IL-2.

## Results

Peripheral blood lymphocytes (PBLs) isolated from human plasma were cultured in the presence (hereafter designated "stimulated") or absence (hereafter designated "resting") of phytohemagglutinin (PHA) for 3 days. PHA-stimulated cells were washed and cultured for an additional 12 hr in the absence of PHA. Both cultures were exposed to varying concentrations of recombinant IL-2 for 15 min, then used to prepare nuclear extracts (see Experimental Procedures). Gel mobility shift assays were performed using a DNA probe derived from the promoter of the gene encoding the FcγRI immunoglobulin receptor that is known to bind Stat proteins avidly (Kotanides and Reich, 1993).

Nuclear extracts prepared from both PHA-stimulated and resting PBLs contained three DNA binding activities capable of forming stable complexes with the FcγRI probe (Figures 1A and 1B). The slowest migrating complex, designated P1, was eliminated when challenged with specific competitor DNA, yet was insensitive to competition by a mutated derivative of the FcγRI probe (see Experimental Procedures). The two more rapidly migrating complexes were insensitive to challenge with specific competitor DNA and therefore deemed nonspecific (NS).

IL-2 treatment of both PHA-stimulated and resting PBL cultures failed to substantially alter the abundance of the activities responsible for generating either the P1 or NS complexes. Cytokine treatment did, however, lead to the appearance of a DNA binding activity, designated P2, that was sensitive to competition by the FcγRI probe (Figures

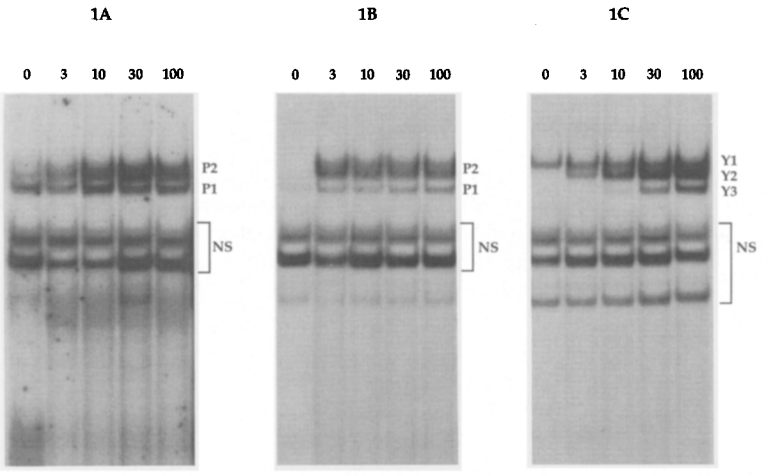



Figure 1. Gel Mobility Shift Assays Using Nuclear Extracts Prepared from Resting and IL-2-Induced Human Lymphocytes

(Left) Results of gel shift assays using nuclear extracts prepared from resting PBLs exposed to varying concentrations of recombinant IL-2. Protein–DNA complexes designated NS were not selectively competed by the FcγRI probe. The P1 and P2 complexes were sensitive to specific competitor but insensitive to a mutated derivative of the FcγRI probe (see Experimental Procedures). The P1 complex was observed irrespective of IL-2 stimulation. The P2 complex was maximally induced in response to intermediate concentrations (10–30 ng/ml) of IL-2.

(Middle) Gel shift results using nuclear extracts prepared from PHA-stimulated PBLs. The pattern of NS, P1, and P2 complexes was similar to that observed from resting PBLs, except for the sensitivity of P2 formation to lower concentrations of IL-2 substantially. (Right) Results of gel shift assays using nuclear extracts prepared from YT cells. Protein–DNA complexes designated NS were not selectively competed by the FcγRI probe. The Y1, Y2, and Y3 complexes were sensitive to specific competitor but insensitive to the mutated FcγRI probe. The Y1 complex was formed with nuclear extracts from unstimulated YT cells, whereas the Y2 and Y3 complexes were induced, respectively, at intermediate and high concentrations of IL-2.

1A and 1B). Optimal activation of the P2 complex in resting PBLs required 30 ng/ml of IL-2. PHA-stimulated PBLs contained peak levels of this activity when stimulated at the lowest dose of IL-2 tested (3 ng/ml).

A transformed human lymphocyte cell line, termed YT (Yodoi et al., 1985), was identified as a potential source for purification of IL-2-induced transcription factors. Nuclear extracts prepared from uninduced YT cells were observed to contain several activities capable of binding to the FcγRI probe (Figure 1C). Competition assays revealed the slowest migrating complex (Y1) to be specific and the more rapidly migrating complexes to be NS. None of the three activities appeared to change as a function of IL-2 presentation. Following exposure for 15 min to recombinant IL-2, however, two new DNA binding activities were observed. One activity, designated Y2, was induced in response to intermediate concentrations of IL-2 (10–30 ng/ml). The other, designated Y3, required exposure to higher concentration of cytokine (30–100 ng/ml). Both of the IL-2-induced DNA binding activities obtained from YT cells were sensitive to specific competitor DNA, yet insensitive to the mutated derivative of the FcγRI probe.

Two observations indicated that the constitutive (P1) and IL-2-induced (P2) complexes obtained from PBLs might correspond, respectively, to the IL-2-induced complexes obtained from YT cells (Y2 and Y3). First, they comigrated when analyzed by the gel mobility shift assay (P1 = Y3 and P2 = Y2). Second, the P2 and Y2 complexes were induced in resting PBLs and YT cells with similar dose dependencies (10–30 ng/ml).

Having obtained provisional evidence that PBLs and YT cells induce similar DNA binding activities in response to IL-2, we set out to purify the polypeptides specifying these activities. Nuclear extracts were prepared from 40 l of YT cells that had been exposed for 15 min to 30 ng/ml of recombinant IL-2. A purification scheme involving ammonium sulfate precipitation followed by S–Sepharose, DNA affinity, and Q–Sepharose chromatography (see Experimental Procedures) led to the isolation of a complex group of polypeptides that migrated on denaturing polyacrylamide gels in the range of 85–100 kDa. Western blotting assays using anti-phosphotyrosine antibodies gave evidence that many, if not all, of these proteins were tyrosine phosphorylated.

The proteins purified according to procedures outlined in the preceding paragraph were cleaved with lysine C and resulting peptide fragments were fractionated by reverse phase capillary high performance liquid chromatography (HPLC). Partial amino acid sequences were resolved for six peptides (see Experimental Procedures). When compared with a data base of known protein sequences, two of the sequenced peptides were found to correspond to Stat1 (Schindler et al., 1992) and four to Stat3 (Akira et al., 1994).

Apparently, YT cells induced with IL-2 contain activated Stat1 and Stat3. Recall, however, that at least three distinct Stat-like complexes were observed in YT nuclear extracts. One such complex, termed Y1, was present irrespective of IL-2 presentation. The other two, designated Y2 and Y3, were induced, respectively, by intermediate and relatively high levels of recombinant IL-2.

To determine the molecular identities of the three complexes, antibodies specific to Stat1 and Stat3 were incubated with nuclear extracts derived from uninduced and IL-2-induced YT cells. As shown in Figure 2, antibodies specific to Stat1 did not affect the Y1 complex formed between uninduced YT nuclear extract and the FcγRI probe. However, when incubated with extracts prepared from cells exposed to 30 ng/ml IL-2, the Stat1 antibody altered the mobility of the most rapidly migrating IL-2-induced complex (Y3). Compared with untreated extracts, or extracts incubated with control antibodies, those incubated with antibodies to Stat1 resulted in a protein–DNA complex that migrated at a significantly retarded (super-

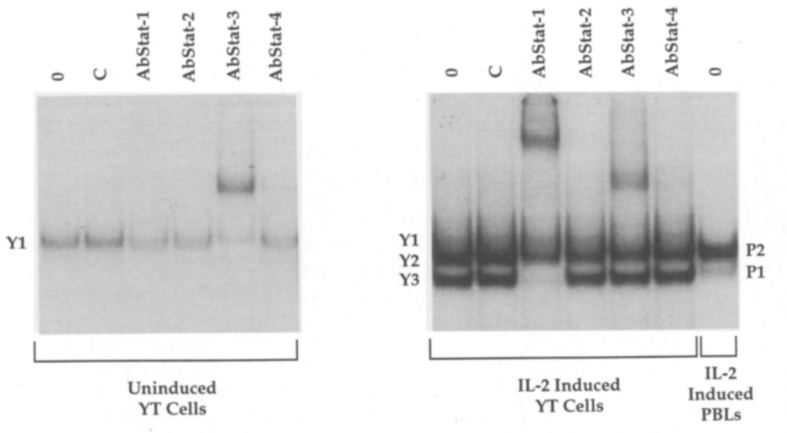



Figure 2. Molecular Identification of Polypeptides Involved in DNA–Protein Complexes Derived from Human Lymphocytes

YT nuclear extracts were prepared from either unstimulated cells (left) or cells that had been exposed to 30 ng/ml of IL-2 (right). Prior to formation of protein–DNA complexes, nuclear extracts were incubated with antibodies to known Stat proteins. Antibodies to Stat3 supershifted the Y1 complex formed by YT nuclear extracts irrespective of IL-2 treatment, indicating the constitutive presence of active Stat3. Antibodies to Stat1 supershifted the Y3 complex formed by YT nuclear extracts following treatment with high levels of IL-2 (see Figure 1). The right lane of the right panel shows protein–DNA complexes formed by nuclear extracts of resting PBLs following induction by 30 ng/ml of IL-2. The more rapidly migrating complex (P1) comigrated with the Y3 complex that reacted with antibodies to Stat1. Antibody supershift assays using PBL nuclear extracts showed that the P1 complex corresponds to Stat1 (data not shown).

shifted) rate. Identical results were observed with nuclear extracts prepared from PBLs induced with 30 ng/ml of IL-2. That is, the P1 complex was selectively supershifted by Stat1 antibodies. We therefore conclude that Stat1 was activated in YT cells in response to relatively high concentrations of IL-2. PBLs also contain Stat1 that, at least in the case of the PHA stimulation, was marginally induced by IL-2.

Antibodies to Stat3 were also tested using nuclear extracts prepared from uninduced YT cells, as well as YT cells and PBLs that had been exposed for 15 min to 30 ng/ml IL-2 (Figure 2). In this case, we observed a distinctive alteration in the migration of the Y1 complex formed by YT nuclear extracts, irrespective of exposure to IL-2. Antibodies to Stat3 did not affect the abundance or migration of DNA binding activities derived from IL-2-induced PBLs, irrespective of stimulation by PHA. According to these observations, we conclude that active Stat3 is constitutively present in YT cells and is not further stimulated by IL-2 in either YT cells or PBLs.

The observations outlined thus far provide a molecular link between Stat1 and a protein–DNA complex induced in YT cells by relatively high levels of IL-2 (designated Y3), as well as a link between Stat3 and a complex present in uninduced YT cells (designated Y1). Left unresolved, however, was the molecular nature of the Y2 activity induced in YT cells by intermediate concentrations of IL-2. A comigrating DNA–protein complex, designated P2, was also induced by intermediate concentrations of IL-2 in resting PBLs, and by very low concentrations of IL-2 in PHA-stimulated PBLs. To resolve the identity of the polypeptides specifying this activity, the complex of Stat proteins purified from YT cells was purged of Stat1 and Stat3 by immunodepletion.

Protein purified from 20 l of IL-2-induced YT cells was incubated sequentially with antibodies specific to Stat1 and Stat3 and Sepharose beads linked to protein A (see Experimental Procedures). Load, flow-through, wash, and bound fractions were then analyzed by SDS–gel electrophoresis and subsequent staining using Coomassie blue as well as antibodies to phosphotyrosine, Stat1 and Stat3. The resulting staining patterns (Figure 3) revealed effective depletion of Stat1 and Stat3. Both Coomassie and anti-phosphotyrosine staining indicated, however, that the

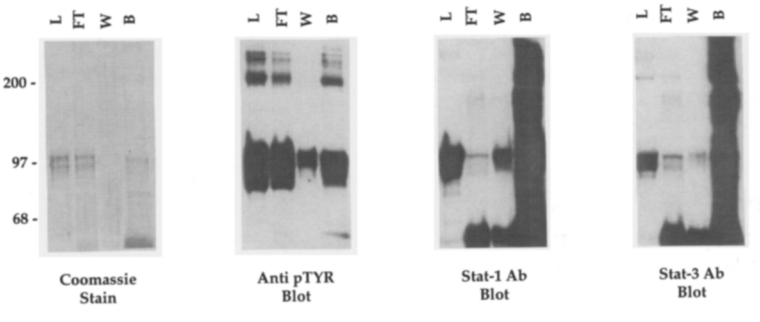
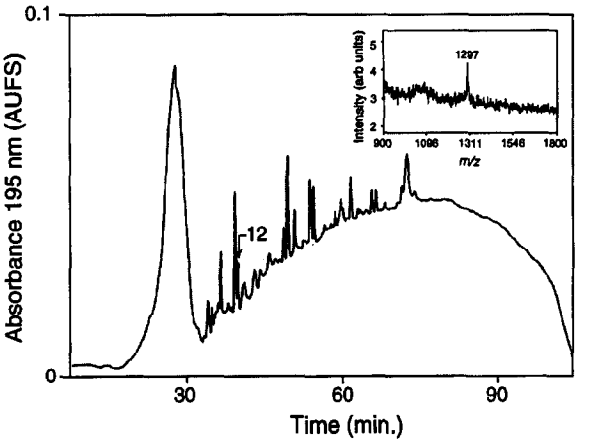



Figure 3. Immunodepletion of Stat1 and Stat3 from Stat-Like Proteins Purified from IL-2-Induced YT Cells

Proteins capable of high affinity binding to the FcγRI probe were purified (see Experimental Procedures) and subjected to immunodepletion using antibodies to Stat1 and Stat3 coupled to protein A–Sepharose. Load (L), flow-through (FT), wash (W), and bound (B) protein fractions were electrophoresed on four SDS–polyacryalmide gels and transferred to PVDF membranes. One membrane (left) was stained with Coomassie blue and the remaining three were stained with antisera specific to phosphotyrosine (second from left), Stat1 (second from right), and Stat3 (right). Note that despite effective removal of Stat1 and Stat3, unbound fraction retained polypeptides migrating with apparent masses of 85–100 kDa that reacted strongly with anti-phosphotyrosine antibodies.




NH2-AVDGXVKPQIK-COOH

Calculated Mass if X is PO4-Tyrosine = 1298.4

Figure 4. MALDI Mass Spectrometry Analysis of a Tyrosine-Containing Peptide Derived from an IL-2-Induced Stat Protein

Large panel shows reversed phase capillary HPLC profile of lysine C peptides digested from the 98 kDa Stat-like protein purified from IL-2-induced YT cells and purged of Stat1 and Stat3 (see Figure 3). Peak 12, which eluted at 41 min, yielded the sequence $NH_2$–AVDGXVKPQIK–COOH. MALDI mass spectrometry analysis of peak 12 yielded a mass of 1,297.0 daltons. The predicted mass of the peptide, if X = phosphotyrosine, is 1,298.4 daltons. A perfect match to this sequence ($NH_2$–AVDGYVKPQIK–COOH) was found in the conceptually translated sequence of the hStat5 cDNA clone (Figure 6).

flow through fraction retained Stat-like proteins. The three most prominent polypeptides remaining following immunodepletion were excised and individually digested with lysine C. Resulting peptide fragments were separated by reverse phase capillary HPLC and subjected to partial amino acid sequence analysis.

The largest of the three polypeptide bands, which migrated with an apparent molecular mass of 98 kDa, yielded three peptide sequences, all of which matched the sequence of a previously characterized protein variously termed mammary gland factor (MGF) or Stat5 (Wakao et al., 1994). Two of the peptides yielded unambiguous sequences of 10 and 14 residues. Both of these sequences were identical to segments of MGF/Stat5 (see Figure 6). The third peptide, designated peak 12 (Figure 4), did not contain an identifiable amino acid residue in the fifth Edman degradation cycle. Of the 11 residues of this sequence, 10 did, however, match perfectly with a segment located close to the carboxyl terminus of MGF/Stat5. The unidentified residue of this peptide corresponded to tyrosine residue 694 of MGF/Stat5, which has been identified as the substrate for Jak-mediated phosphorylation (Gouilleux et al., 1994).

Reasoning that the ambiguity observed upon amino acid sequence analysis of this third peptide might reflect the fact the fifth residue was phosphorylated on tyrosine, and therefore not extracted by the nonpolar solvent used to extract the phenylthiohydantion during Edman degradation (Aebersold et al., 1991), we performed matrix-assisted laser ionization desorption/ionization (MALDI) mass spectrometry analysis (Figure 4). The observed mass of the peptide (1,297.0 ± 2 daltons) corresponded very closely to that predicted for the tyrosine phosphoform of the sequence $NH_2$–AVDGYVKPQIK–COOH (1,298.4). Given that the affinity chromatographic step of our purification procedure selects for the active form of Stat proteins (Hou et al., 1994), and that the Stat activation cycle entails tyrosine phosphorylation (Darnell et al., 1994), it is not surprising that the Stat protein activated in IL-2-induced YT cells is tyrosine phosphorylated.

Peptide sequence analysis of two smaller Stat-like proteins remaining after immunodepletion (migrating with apparent masses of 94 and 88 kDa) also yielded sequences identical to MGF/Stat5. In both cases, peptides bearing the ambiguous sequence $NH_2$–AVDGXVKPQIK–COOH were also observed. We thus conclude that the sample of proteins purified from IL-2-induced YT cells, following depletion of Stat1 and Stat3, is largely composed of polypeptides related to MGF/Stat5. Moreover, the three predominant species (98, 94, and 88 kDa) all appear to be tyrosine phosphorylated on the same residue. We tentatively conclude that activation of this family of proteins by IL-2 in human YT cells entails tyrosine phosphorylation at the same position as prolactin-mediated activation of MGF/Stat5 (Gouilleux et al., 1994).

Having established a molecular link between Stat5 and the DNA binding activity induced by IL-2 in YT cells (designated Y2), we set out to determine the identity of the DNA binding activity induced by IL-2 in PBLs. Whereas the activity induced by IL-2 in both resting and PHA-stimulated PBLs (designated P2) comigrated on nondenaturing gels with that induced in YT cells (Y2), we did not have antibodies that would allow rigorous testing of the identity of the Stat protein induced in PBLs. As such, we purified the activity to obtain partial amino acid sequence of the relevant polypeptides.

Lymphocytes were isolated from 10 U of human blood and grown for 3 days in the presence of PHA. The cells were then washed, cultured for 12 hr in the absence of PHA, exposed for 15 min to 10 ng/ml of IL-2, harvested, and used to prepare nuclear extracts. The IL-2-induced DNA binding activity was purified by the same method used to isolate Stat proteins from YT cells (see Experimental Procedures).

As shown in Figure 5, these procedures led to the purification of two polypeptides that migrated with apparent molecular masses of 96 and 95 kDa. Western blot assays provided evidence that both of these polypeptides reacted strongly with antibodies to phosphotyrosine. Each polypeptide was excised, digested with lysine C, and the resulting peptide fragments were fractionated by capillary HPLC. The two proteins yielded very similar lysine C digests as assessed by the UV spectral traces of the resulting chromatograms. Mass spectrometry analysis of individual coeluting peptides provided additional evidence of a close relatedness between the 96 and 95 kDa polypeptides. Two peptides were subjected to gas phase sequenc-

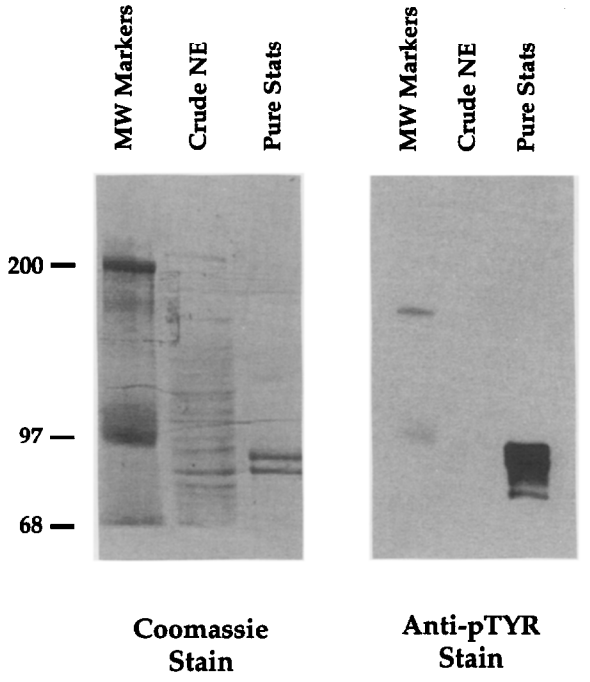



Figure 5. Purification of Stat-Like Proteins from IL-2-Induced PBLs

PHA-stimulated PBLs were exposed to 10 ng/ml IL-2 and used to prepare nuclear extracts. IL-2-induced proteins capable of avid interaction with the FcγRI probe were purified (see Experimental Procedures), subjected to SDS–gel electrophoresis, transferred onto a PVDF membrane, and stained with anti-phosphotyrosine antibodies. Following development of the Western blot (right), the filter was stained with Coomassie blue (left). Two prominent phosphotyrosine-containing polypeptides were observed to migrate with apparent molecular masses of 90 and 88 kDa. Partial amino acid sequencing of both polypeptides revealed multiple fragments bearing sequences identical to hStat5 (see Figure 6).

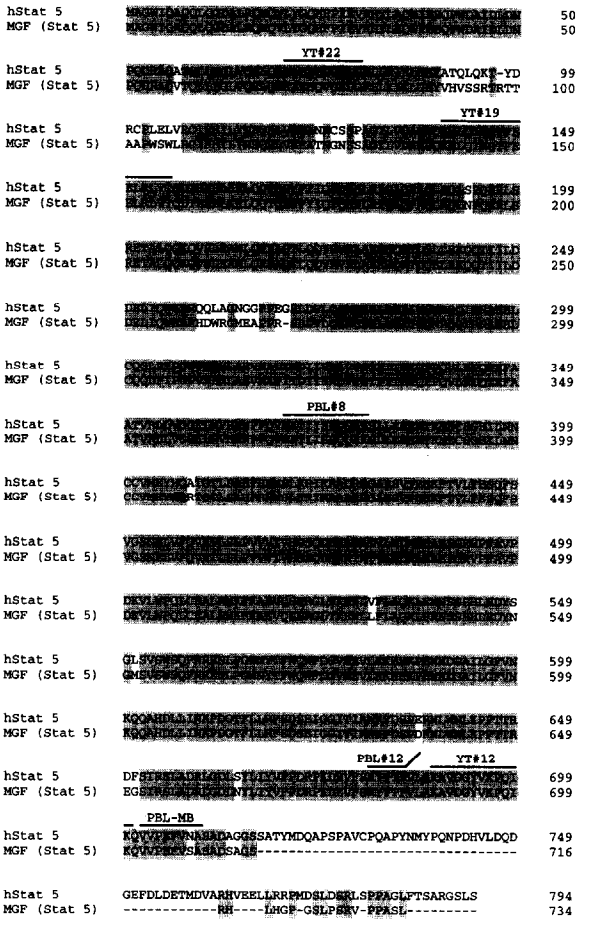



Figure 6. DNA Sequence Comparison Between hStat5 and MGF

A recombinant clone encoding hStat5 was isolated from a cDNA library prepared with mRNA from human umbilical vein endothelial cells. DNA sequence was resolved on both DNA strands by the chain termination method of DNA sequencing. Conceptually translated amino acid sequences of hStat5 (top) and MGF (bottom) are compared (Wakao et al., 1994). Overlined segments correspond to peptides sequenced from protein purified from IL-2-induced YT cells (YT 22, YT 21, and YT 12) and IL-2-induced PBLs (PBL 12 and PBL–MB). The hStat5 ORF consists of 794 aa that, in an unmodified state, specifies the monomeric size of 90.5 kDa. Abbreviations for the amino acid residues are the following: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L. Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

ing, yielding sequences highly related to MGF/Stat5 (see Figure 6). We thus conclude that the predominant Stat protein induced by IL-2 in PHA-stimulated human lymphocytes is highly related to MGF/Stat5.

A cDNA clone corresponding to the human form of Stat5 was isolated and sequenced (Figure 6). Conceptual translation of the DNA sequence specified an open reading frame (ORF) of 794 aa residues which, in an unmodified state, predicts a molecular mass of 90,544 daltons. Segments identical to all three of the peptides derived from the IL-2-induced protein from YT cells (Y2) and two of the peptides derived from the IL-2-induced protein from PBLs (P2) were observed in the ORF. The third peptide sequence derived from the IL-2-induced protein purified from PBLs matched the ORF at 5 consecutive residues, yet diverged significantly at amino acid residue 687. We speculate that this may represent the result of alternative splicing or presence of a second highly related hStat5 gene. Additional studies will be required to resolve between these alternative hypotheses.

Figure 6 compares the sequence of the cloned gene product, hereby designated human Stat5 (hStat5), and the previously characterized MGF protein of sheep (Wakao et al., 1994). Whether the two proteins represent molecular counterparts can not be conclusively established at present. Although significant segments of amino acid sequence identity occur throughout the two proteins, three divergent regions were observed (residues 90–107, 260–272, and 717–COOH). As such, it is possible that the sheep and human proteins are encoded by two distinct genetic loci.

The tissue distribution of hStat5 gene expression was examined by Northern blotting as shown in Figure 7. Messenger RNAs of measuring roughly 3, 4, and 6 kb in length were observed. The larger two mRNAs were observed in all human tissues that were examined, with roughly equivalent levels occurring in placenta, skeletal muscle, spleen, thymus, prostate, testis, ovary, small intestine, colon, and

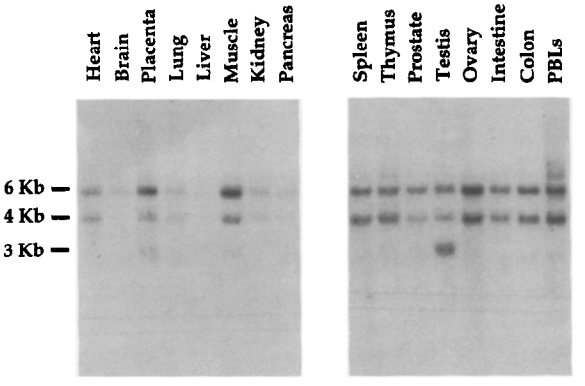



Figure 7. Tissue Distribution of hStat5 Messenger RNA

Nylon membranes containing polyadenylated-enriched mRNA from 16 human tissues were probed by nucleic acid hybridization at high stringency for hStat5 mRNA. Two prominent bands roughly 4 and 6 kb in length were observed in most tissues. A smaller band, approximately 3 kb in length, was observed in placenta, skeletal muscle, and kidney.

PBLs; slightly lower levels in heart, lung, kidney, and pancreas; and considerably lover levels in brain and liver. The small (3 kb) mRNA was observed in a more restricted pattern, limited to placenta, skeletal muscle, and testis. It is unclear whether these various hStat5 mRNAs are expressed as differentially processed products of a single gene or the transcribed products of distinct genes.

## Discussion

Here we report the purification and molecular identification of transcription factors activated in human lymphocytes in response to IL-2. Our studies have made use of a transformed natural killer–like cell line, termed YT (Yodoi et al., 1985), as well as resting and PHA-stimulated PBLs. Uninduced YT cells were observed to contain a Stat-like DNA binding activity that, upon purification and molecular characterization, was identified as the human form of Stat3. Previous studies have identified Stat3 as a transcription factor rapidly activated by IL-6 (Akira et al., 1994). We hypothesize that cultured YT cells secrete an autocrine activity responsible for the constitutive presence of activated Stat3. Treatment of YT cells with IL-2 did not appear to influence the abundance of activated Stat3. Likewise, IL-2 did not lead to the induction of Stat3 in either resting or PHA-stimulated PBLs.

One prominent Stat-like activity was observed to be induced by IL-2 in all of the cell cultures hereby examined. This activity, designated Y2 in YT cells and P2 in resting and PHA-stimulated PBLs, was identified as hStat5, a human protein bearing significant similarity to MGF of sheep (Wakao et al., 1994). Optimal induction of hStat5 in YT cells and resting PBLs required 10–30 ng/ml of IL-2. Activation of hStat5 in PHA-stimulated PBLs required no more than 3 ng/ml cytokine. Whereas we have not resolved the molecular basis for this observed change in sensitivity to IL-2, it may result from an increase in the concentration of one or more of the subunits constituting the high affinity IL-2 receptor.

Several observations made in this study give evidence of heterogeneous forms of hStat5. First, regardless of whether purified from YT cells or PHA-stimulated PBLs, hStat5 consisted of multiple polypeptides. Second, Northern blot assays of mRNA prepared from a variety of human tissues revealed three distinct hStat5 transcripts. Finally, amino acid sequence analysis of the 95 kDa polypeptide purified from IL-2-stimulated PBLs revealed a peptide that matched the conceptually translated hStat5 sequence over 5 residues, yet diverged significantly over the contiguous 7 residues. It may be important that the location of this divergence is 8 residues upstream from the identified site of tyrosine phosphorylation. If correct, the sequence of this peptide predicts the presence of hStat5 variants that may be tyrosine phosphorylated in different ways. The origin of this variation may reflect differential splicing of transcripts encoded by a single hStat5 gene, or the presence of more than one encoding gene.

A second Stat-like activity was induced in two of the three cell types. This activity, designated Y3 in YT cells and P1 in resting and PHA-stimulated PBLs, was identified as the human form of Stat1. Induction of Stat1 in YT cells required very high concentrations of IL-2. Low concentrations of IL-2 resulted in a modest induction of Stat1 in PHA-stimulated PBLs. Finally, resting PBLs failed to induce Stat1 substantially in response to IL-2.

Partial amino acid sequence of the Stat protein (hStat5) activated at the lowest concentration of IL-2 provided indirect evidence of tyrosine phosphorylation at residue 694. Edman degradation of the peptide bearing this tyrosine failed to yield an amino acid at the expected cycle. If this tyrosine residue was quantitatively phosphorylated, it would not be expected to be extracted by the nonpolar solvents used for amino acid sequence analysis (Aebersold et al., 1991). Additionally, mass spectrometry analysis of the relevant peptide yielded a mass consistent with the tyrosine phosphoform (Yip and Hutchens, 1992). On the basis of these observations, we conclude that IL-2 activation of hStat5 entails tyrosine phosphorylation at precisely the same position as prolactin-mediated activation of MGF (Gouilleux et al., 1994).

The relevance of the aforementioned finding may be signified by the fact that prolactin and IL-2 activate different Jak kinases. Two recent studies have provided evidence that prolactin activates Jak2 (Gilmour and Reich, 1994; Rui et al., 1994). In contrast, IL-2 has been shown to activate Jak1 and Jak3 (Boussiotis et al., 1994; Johnston et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). Having found that MGF and hStat5 are tyrosine phosphorylated on the same residue (694), we provisionally conclude that the specificity of activation is not controlled by Jak kinases. We instead believe that the functional Jak employed in Stat activation is dictated by the intracellular domain of the relevant receptor. In the case of the prolactin receptor, MGF activation is mediated by Jak2, owing, presumably, to the affinity of Jak2 to the intracellular domain of the prolactin receptor. In contrast,

hStat5 is activated by Jak1, Jak3, or both by virtue of their affinity to one or more chains of the IL-2 receptor. Taken most literally, this interpretation predicts that specificity of Stat activation rests on coupling of the latent transcription factor to the intracellular domain of its cognate receptor (Greenlund et al., 1994; Hou et al., 1994). If so, the decoding of this hypothetical network of receptor coupling represents a critical and central objective for the field.

The observations reported herein raise several important questions. First and foremost, how is it that IL-2 is able to coordinate a selective biological response by activating transcription factors that are subject to regulation by physiologically distinct cytokines and growth factors? Firm evidence has demonstrated activation of Stat1 by cytokines, such as interferon γ, as well as growth factors, such as epidermal growth factor, that are biologically distinct from IL-2. Likewise, MGF, the putative sheep counterpart of hStat5, is activated by a seemingly distinct stimulant (prolactin) from IL-2. If different cytokines and growth factors activate overlapping or identical Stat proteins, how are biologically distinct patterns of response generated?

It is possible that the concentration of IL-2 required for activation of Stat1 (30–100 ng/ml in YT cells) is not physiologically relevant, and that Stat1 does not play a significant role in mediating the biological effects of IL-2. Given that hStat5 is activated at substantially lower cytokine concentrations, we judge it to be a more likely candidate for transmitting the effects of IL-2. In this regard, it is intriguing that prolactin and IL-2 do share the capacity to stimulate proliferation of T lymphocytes (Kelly et al., 1991). Thus, although at an organismal level IL-2 and prolactin coordinate largely distinct functions, they may act through a common molecular pathway in T lymphocytes by activating hStat5. Indeed, Gilmour and Reich (1994) have provided evidence that prolactin and IL-2 induce an indistinguishable DNA binding activity in a rat T lymphoma cell line termed Nb2.

A second issue left unresolved by the present study concerns possible linkage between the various chains of the IL-2 receptor and the Stat proteins activated in response to ligand. IL-2 has been shown to activate Jak1 and Jak3 (Boussiotis et al., 1994; Johnston et al., 1994; Miyazaki et al., 1994; Russell et al., 1994; Witthuhn et al., 1994). Since it has been firmly established that Jak kinases play a central and direct role in Stat activation (Darnell et al., 1994), one might anticipate transient association of IL-2-induced Stat proteins with one or more components of the IL-2 receptor. Studies relevant to this issue have provided evidence that Stat1 may transiently couple to a phosphotyrosine located within the intracellular domain of the interferon-γ receptor at an early point in its activation cycle (Greenlund et al., 1994). Similar interpretations have been concluded from studies of IL-4-mediated Stat activation (Hou et al., 1994). If receptor coupling proves to a broadly utilized step in the Stat activation cycle, it is predictable that a docking site for hStat5 will be discovered within the intracellular domain of one or more of the IL-2 receptor subunits. We have preliminarily observed that two tyrosine-phosphorylated peptides derived from the C-terminal domain of the IL-2Rβ receptor chain are capable of inhibiting the DNA binding activity of hStat5 (J. H. and S. L. M., unpublished data). Such observations, coupled with recent data showing that mutated derivatives of the IL-2Rβ receptor chain lacking the two most C-terminal tyrosine residues fail to activate hStat5 (H. Fujii, Y. Nakagawa, U. S,, A. Kawahara, F. Gouilleux, B. Groner, J. N. Ihle, Y. Minami, T. Miyazaki, and T. Taniguchi, personal communication), are indeed consistent with the direct receptor coupling model that has emerged from studies of interferon-γ (Greenlund et al., 1994) and IL-4 (Hou et al., 1994).

## Experimental Procedures

### Cell Culture

The transformed human lymphocyte cell line YT (Yodoi et al., 1985) was grown in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 10mM HEPES (pH 7.2), 10 μM β-mercaptoethanol, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 μg/ml ampicillin. Cells grown to a density of 7 × $10^5$/ml in T-150 flasks were exposed to recombinant IL-2 (Biosource International) for 15 min at 37°C, harvested by centrifugation, and used to prepare nuclear extracts (Dignam et al., 1983). PBLs obtained from Irvin Memorial Blood Bank (San Francisco, California) were harvested by centrifugation, resuspended in phosphate-buffered saline, and applied atop Lymphoprep (Nycomed AS) in 50 ml conical centrifuge tubes. Following centrifugation, the lymphocyte layer was retrieved, washed, and resuspended in the same culture medium used for growth of YT cells. Cells were grown either in the presence or absence of Phaseolus vulgaris lectin (PHA) (Sigma) for 3 days. PHA-stimulated cells were washed, resuspended in culture medium lacking PHA, and grown for 12 hr. IL-2 treatment and preparation of nuclear extracts was the same as used for YT cells.

### Purification of IL-2-Induced Transcription Factors

Nuclear extracts from YT cells (1 g) and PBLs (0.25 g) were precipitated with 30% ammonium sulfate. The proteins were then removed by centrifugation and the supernatant was then treated with 60% ammonium sulfate. Proteins precipitating between 30%–60% ammonium sulfate were recovered by centrifugation, resuspended in 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonate, 0.5 mM dithiothreitol (DTT), 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM benzamidine, 1 mM sodium vanadate, 1 mM NaF, 5 mM β-glycerolphosphate (buffer C), and dialyzed against buffer C supplemented with NaCl to a final concentration of 150 mM.

After dialysis, insoluble proteins were removed by centrifugation and the remaining material was chromatographed over a 100 ml S–Sepharose (Pharmacia) column. Protein flow-through was mixed with a DNA-affinity resin prepared by coupling synthetic, biotinylated DNA corresponding to the IL-4 response element of the gene encoding FcγRI (5′-GTATTTCCCAGAAAAGGAAC-3′) to streptavidin agarose (Sigma). After binding (2 hr at 4°C), the affinity matrix was placed on a disposable column and washed sequentially with 10 ml of buffer C, 4 ml of buffer C supplemented with a mutated variant of the IL-4 response element (5′-GTATCACCAGTCAAGGAAC-3′) at 0.2 mg/ml, and 10 ml of buffer C. Protein was eluted by exposure to buffer C supplemented with 0.35 M NaCl, dialyzed against buffer C, and placed on a 0.5 ml Q–Sepharose (Pharmacia) column. The column was washed with 5 ml of buffer C and protein was eluted with 1 ml of buffer C supplemented with 0.35 M NaCl.

### Partial Amino Acid Sequencing of IL-2-Induced Transcription Factors

Purified IL-2-induced DNA binding proteins were subjected to SDS–gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was stained with Coomassie blue R-250 in 40% methanol and 0.1% acetic acid for 30 s, then destained for 5 min with 10% acetic acid in 50% methanol. Relevant polypeptides were excised from the membrane and alkylated with isopropylaceta-

mide (Krutzsch and Inman, 1993; Henzel et al., 1994) and then digested in 50 μl of 0.1 M ammonium bicarbonate, 10% acetonitrile with 0.2 μg of lysine C (Wako) at 37°C for 17 hr. The solution was then concentrated to 20 μl and directly injected onto a capillary high performance liquid chromatogram. The HPLC consisted of a prototype capillary gradient HPLC system (Waters Associates) and a model 783 UV detector equipped with a Z-shaped flow cell (LC Packings, Incorporated). A 30 cm length of 0.025 mm inner dimension glass capillary was connected to the outlet of the Z-shaped cell inside the detector housing to minimize the delay volume. The total delay volume was 0.45 μl, which corresponded to a delay of 6 s for a flow rate of 3.5 μl/min. The short delay greatly facilitated hand collection of individual peptide peaks. Peptides were separated on a C18 capillary column (0.32 by 100 mm) (LC Packings, Incorporated) developed with 0.1% aqueous trifloracetic acid as buffer A and acetonitrile containing 0.07% trifluoroacetic acid as buffer B. Isolated peptides were sequenced on a 490 Applied Biosystems sequencer. Residual peptides retained on the PVDF membrane were also subjected to Edman sequencing. In the case of hStat5 purified from PHA-stimulated PBLs, this provided partial amino acid sequence of a very large lysine C peptide, designated PBL–membrane bound (MB) colinear with the C terminus of the intact hStat5 protein. The same sequence was found for this peptide regardless of whether it was derived from the 96 or 95 kDa polypeptides (see Figure 5). Sequence interpretation was performed on a DEC 5900 computer (Henzel et al., 1987).

### Immunodepletion, Supershifting, and Oligonucleotide Competition Assays

Protein–DNA complexes were visualized by a gel mobility shift assay under nondenaturing conditions. Specificity of protein–DNA interaction was tested using 100-fold molar excess of either the native FcγRI probe (5′-GTATTTCCCAGAAAAGGAAC-3′) or the mutated derivative (5′-GTATCACCCAGTCAAGGAAC-3′). Antibody supershift experiments were performed by incubating protein samples with antibodies (Santa Cruz Biotech) for 30 min at 4°C prior to exposure to the FcγRI DNA probe. Proteins purified from YT cells were purged of Stat1 and Stat3 by immunodepletion. Of each antibody, 500 μg (specific to Stat1 and Stat3) was incubated for 2 hr at room temperature with a slurry of protein A–Sepharose beads (Pharmacia) sufficient to yield 100 μl of bed volume. Beads were washed three times with 1 ml of buffer C and then incubated for 2 hr at room temperature with Stat proteins purified from IL-2-induced YT cells. Beads were recovered by centrifugation and washed three times with 1 ml of buffer C. Unbound, wash, and bound fractions were recovered and subjected to SDS–gel electrophoresis for subsequent staining with Coomassie blue, anti-phosphotyrosine antibodies, and Stat antibodies.

### Mass Spectrometry

Aliquots (0.2 μl) of the capillary HPLC-purified lysine C peptides were mixed on the sample probe tip with 0.2 μl of α-cyano-4-hydroxycinnamic acid (saturated in 50% acetonitrile 2% trifluoroacetic acid). MALDI mass spectra were obtained with a Vestec (Houston, Texas) LaserTec Research laser desorption linear time-of-flash mass spectrometer equipped with a 337 nm VSL-337 ND nitrogen laser (Laser Sciences, Incorporated).

### Cloning of hStat5 cDNA

A cDNA library prepared from human umbilical vein endothelial cells was probed at low stringency with a fragment of the IL-4 Stat cDNA (Hou et al., 1994) corresponding to the SH3 and SH2 domains of the protein. Radiolabeled probe DNA was hybridized with filter lifts at 42°C in a solution containing 20% formamide, 10× Denhardt's solution, 5× SSPE, 0.5% SDS, and 100 μg/ml salmon sperm DNA. After hybridization (16 hr), filters were washed twice at 42°C in a 2× SSPE, 0.5% SDS, and twice at 42°C in 2× SSPE. Roughly equivalent numbers of clones encoding IL-4 Stat and hStat5 were recovered. A 3.8 Kb hStat5 cDNA clone was sequenced on both DNA strands using an Applied Biosystems automated DNA sequencer.

### Northern Blotting

RNA blot hybridization with a uniformly labeled DNA probe prepared from the hStat5 cDNA clone and multiple tissue Northern blot membranes (Clone-Tech) were used. The probe DNA fragment corresponded to the region of the hStat5 cDNA that encodes the amino terminal segment of the protein (excluding the putative SH3 and SH2 domains). Probe labeling, hybridization, and membrane washing were performed as described (Sambrook et al., 1989).

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**Note Added in Proof**

After submission of this work the authors were provided antibodies to MGF by Drs. B. Groner and F. Gouilleux. When used in gel mobility shift assays, antibodies to the sheep MGF protein selectively supershifted the p2 Stat protein complex induced by IL-2 in PHA-stimulated peripheral blood lymphocytes, further confirming the significant relatedness between hStat5 and MGF. The authors thank Drs. Groner and Gouilleux for the generous provision of antisera to MGF.

**GenBank Accession Number**

The GenBank accession number for hStat5 as reported in this paper is L41142.